DEVELOPMENTAL STUDIES
AND
LABORATORY INVESTIGATIONS
Conducted by
Veterinary Services Diagnostic Laboratories
Fiscal Year 1973

Animal and Plant Health Inspection Service
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DEVELOPMENTAL STUDIES AND LABORATORY INVESTIGATIONS CONDUCTED BY VETERINARY SERVICES DIAGNOSTIC LABORATORIES FISCAL YEAR 1973

ANAPLASMOSIS

PRESERVATION OF SERUM SAMPLES FOR THE ANAPLASMOSIS MICROTITER COMPLEMENT-FIXATION TEST. Blackburn, B. O., Wright, H. S. and Sherman, K. C. (Project Report)

Introduction

The "Standardized Complement-Fixation Test for Anaplasmosis" published by the Animal and Plant Health Inspection Service requires the addition of aqueous phenol to serum samples that are submitted for testing. In the recent procedures described for conversion to the microtiter technique, this provision was retained. Since the Veterinary Services Diagnostic Laboratory (VSDL), Ames, Iowa, now has responsibility for all export tests, it would be convenient to drop the phenol requirement. One serum sample could then be used for the various serologic tests required. This study was designed to determine whether serum not treated with phenol could be used effectively in the microtiter complement fixation test for anaplasmosis.

Materials and Methods

Serum samples.—Ten serum samples were obtained from known positive animals at the Oklahoma State University Veterinary Research Farm, Pawhuska, Okla. Ten negative serum samples were obtained from animals in the National Animal Disease Laboratory herd. Each serum sample was divided into four parts and each part randomly assigned one of four treatments as follows: (A) refrigeration, (B) refrigeration for 5 days followed by adding aqueous phenol to a final concentration of 0.5 percent, (C) aqueous phenol added to a final concentration of 0.5 percent and (D) freezing. In effect, this gave 20 serum samples by each treatment.

Test procedures.—Serum samples that had been kept refrigerated were tested first. In preparation for the test, each of the 20 samples was further divided to form 25 replicate subsamples. Thus, the total number of subsamples for the treatment group was 500. These subsamples were randomly ordered by using a random number table. The subsamples were then tested following the procedures described by the Beltsville laboratory ("A Microtiter Technique for the Complement Fixation Test for Anaplasmosis"). This same procedure was used for testing the samples receiving the other three treatments.

Results

Results following the screening test (serum dilution 1:5) are summarized in table 1. None of the known negative subsamples gave positive reactions and only one of the known positive subsamples gave a negative reaction. The number of suspicious reactions among the known negatives was higher in the phenol treatment group than in any of the other treatment groups. None of these differences are statistically significant.

The titration results for the known positive subsamples are presented in table 2. Only one of the known positive subsamples titrated had an antibody titer less than 1:20 and this subsample was in the phenol treatment group.

Table 1.—Results of CF tests following four different serum treatments

Source	Treatment	CF test results			
		Positive	Suspect	Negative	No test
Known Positive Animal	Phenol	248	0	0	2
	Refrigeration	248	0	0	2
	Refrig. and Phenol	249	0	1	0
	Freezing	250	0	0	0
Known Negative Animal	Phenol	0	8	241	1
	Refrigeration.................	0	3	248	3
	Refrig. and Phenol	0	· 7	243	0
	Freezing	0	6	244	0

Table 2.—CF titers of known positive serum subsamples following specified treatments for preservation

Treatment	20 or less	40	Titer[1]		320	640 or more
			80	160		
(A) Refrigeration[2]	6	41	69	74	32	6
(B) Refrig. and Phenol	9	61	82	72	23	3
(C) Phenol (Standard)	52	70	71	47	9	1
(D) Freezing	20	85	64	66	11	4

[1] Titer expressed as reciprocal of serum dilution giving 4+ reaction.
[2] Titration results for 22 of the 250 subsamples were invalid because of mechanical error.

Titers following the different treatments were compared using the X^2 test. Calculated X^2 values for the comparisons are presented in table 3. Each of the three experimental treatments (A, B and D) resulted in titers significantly higher than the standard phenol treatment (C). Also, titers following treatments A and B were significantly higher than titers following treatment D. The least difference detected was between treatments A and B.

Table 3.—Calculated X^2 values on comparison of titers between treatments[1]

Treatment comparison	X^2 value	P[2]
A vs C	65.71	<0.005
B vs C	44.10	<0.005
D vs C	21.23	· <0.005
A vs B	7.04	<0.25
A vs D	32.12	<0.005
B vs D	14.84	<0.025

[1] A = Refrigeration.
   B = Refrigeration for 5 days followed by adding phenol.
   C = Phenol.
   D = Freezing.
[2] P = 0.05 or less was considered significant.

Discussion and Conclusion

It appears that the experimental serum preservation methods used were superior to the standard preservation method. This conclusion is drawn because in each case the antibody titers were higher following the experimental treatments and there was no significant increase in false positives, false negatives or suspects. The one false negative subsample appeared to be due to an error in preparing the subsamples.

Perhaps a weakness of this study was the fact that no low titered (1:5) serum was included among the known positive samples. Animals with such titers have been proven to be carriers of *Anaplasma marginale*. However, one would logically expect that low titered serums would show the same effect from the experimental treatments as the serums used in the study. This would possibly enhance the ability of the test to detect low titered carriers.

The degree of variation observed in titers following the different treatments was greater than expected and the comparisons made indicate that one or more influential factors were not controlled. The information obtained was not sufficient to determine definitely the identity of these factors. However, two variables were noted which could have been involved. First, samples in treatment groups A and B were less hemolyzed than those in groups C and D. Second, the technique for mixing serum and phenol was different for samples in treatment groups B and C. Samples in treatment group C were cold (approximately 4° C.) and the phenol was added to the serum. For treatment group C, the serum was at room temperature and was added to the phenol.

There is no evidence from the results obtained that phenol preservation of serum samples is necessary or even desirable.

3

BLUETONGUE

in sheep

BLUETONGUE VIRUS STUDIES. I. PROPAGATION IN CELL CULTURES AND THE EFFECTS OF FREEZING. Carbrey, E. A., Gustafson, G. A. and Lee, L. R. (Project Report)

Summary

Coverslip cell cultures were inoculated with blood from bluetongue (BT) infected sheep in the acute stages of the disease. Infected cells were detected by immunofluorescence in embryonic ovine kidney and embryonic bovine tracheal cells 5 to 7 days later. Negative results were obtained following inoculation of embryonic bovine kidney, embryonic ovine testes, and bovine turbinate cells. However, propagation of BT virus was successful on all primary and cell line cultures when the inoculum was mouse brain adapted virus in 10 percent brain tissue suspension.

There was a hundredfold loss of titer in BT virus infected sheep blood that was stored at $-20°$ C. for one year.

Introduction

Isolation and identification of bluetongue virus in the laboratory has been hampered by the lack of a suitable cell culture system. Bluetongue (BT) virus obtained directly from the blood of an infected sheep does not readily propagate in cell cultures. Some passages of the virus in the chick embryo (CE) or mouse brain seem to be required and the virus becomes cytopathogenic after adaptation.

Haig *et al* (7)[1] were unable to grow BT virus on lamb kidney cell cultures from the serums of infected sheep. However, after one passage in the CE, a cytopathic effect was detected by the fifth day after inoculation. If four passages were made in the CE, then the cytopathic effect was well marked by the third day of growth in the lamb kidney cells. Fernandez (6), however, was able to culture BT virus on lamb kidney cells directly from the viremic blood of acutely ill sheep. A cytopathic effect was detected as early as 24 to 36 hours.

Pini *et al* (11) were the first to describe a fluorescent antibody technique for detecting BT virus infected cells in coverslip cultures. The degree of fluorescence was high with concentrated cell culture adapted virus but quite low when the virus source was blood from infected sheep. Baby hamster kidney cells were satisfactory when horse serum was used in the medium after the cells were exposed to the virus. They (10) also found that the fluorescent antibody reaction was group specific since cells infected with six different antigenic types of the virus were stained with the same conjugate.

Bowne and Jochim (2) obtained specific viral fluorescence in lamb kidney cell cultures 28 hours after inoculation. However, the BT virus was isolated initially by the intravenous inoculation of the CE and then passaged six times in lamb kidney cell culture. The conjugate was prepared from goat serum.

Ruckerbauer *et al* (12) described the use of the fluorescent antibody technique for the detection of BT virus in bovine fetal kidney cell cultures inoculated with blood and tissues from infected sheep. The technique was also applied to frozen cultures and tissue impressions with some degree of success.

After preliminary passage of BT virus six times in lamb kidney cells, Ohder *et al* (9) were able to detect the fluorescence of infected baby hamster kidney cells in 26 hours. A cytopathic effect was detected at 72 hours when 30 percent of the cells were fluorescing. The fluorescent antigen appeared granular and was found in the cytoplasm.

It was considered of value to investigate the use of cell cultures for the initial isolation and detection of BT virus by immunofluorescence.

A related diagnostic problem is the lack of information on the stability of BT virus at temperatures below $0°$ C. Since an effort is made to have all specimens for virus isolation frozen and shipped to the laboratory packed in dry ice, it is important to know if an exception should be made for BT virus. It was reported that the BT virus could

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[1] Number in parentheses refers to References at the end of this report.

be stored frozen at extremely low temperatures such as −78° C. but not at the temperature of the ordinary household freezer, −20° C. An experiment was performed to determine the stability of BT virus at −20° C.

Materials and Methods

Virus Strains.—Bluetongue virus strain California BT8, 10th sheep passage was obtained by bleeding an infected sheep 6 DPI. It was supplied[2] in an anticoagulant preservative solution, OPG.[3] California (type 10) BT virus, mouse brain passage was obtained as freeze dried mouse brain.[4]

Fluorescent Antibody Conjugate.—Sheep 877 was inoculated with 2.0 ml of BT virus suspension, strain BT8, blood with OPG. Serum was collected 54 DPI when the complement fixation (CF) titer was 1-40 and a conjugate was prepared as previously described. (8, 13)

Experimental Animals.—Sheep were obtained from two closed flocks located in Iowa that had been tested and found negative for BT by the CF test. All sheep were tested before inoculation and found negative. Sheep used for virus detection were maintained in plastic isolation cages maintained under negative air pressure. One group of three sheep employed for long-term studies were kept together in an isolation room free of insect vectors.

Complement Fixation Test.—The CF test was performed according to the method of Boulanger as previously described (1).

Cell Cultures.—Primary embryonic bovine kidney (EBK), embryonic ovine testes (EOT), and embryonic ovine kidney cell cultures (EOK); and bovine turbinate (BT) and embryonic bovine tracheal (EBTr) cell lines were propagated on coverslips in Leighton tubes employing appropriate cell culture mediums as previously described (5, 3). The culture medium was decanted and virus inoculum consisting of heparinized whole blood or 10 percent mouse brain suspension was placed on the cell sheets. The cultures were maintained at 37° C. for at least 2 hours, washed three times with Earle's balanced salt solution, and the nutrient medium was replaced. The coverslip cultures were kept at 37° C. and at daily intervals selected coverslips were harvested and strained with the conjugate (4).

Storage of Frozen Virus.—The virus inoculums were maintained as appropriate at −20° C. and −78° C.[5]

Results

Cell Culture of Bluetongue Virus From Sheep Blood and Mouse Brain.—Heparinized whole blood samples were obtained from infected sheep during the clinical stages of the disease usually 4 - 10 DPI, and isolation attempts were made on cell cultures. Coverslip cultures were stained daily from exposed EBK, EOT, EOK, BT and EBTr cell cultures. Fluorescing cells were detected on coverslips of EOK cell cultures that had been incubated for 5 to 6 days after exposure to virus blood. The fluorescing viral antigen was granular in appearance and found in the cytoplasm of the infected cells. However, the detection of infected cells by immunofluorescence was not consistent. A variety of methods were employed to improve the replication of virus recovery in the primary EOK cultures without success.

Bluetongue virus from infected sheep blood was isolated only once on EBTr cell cultures and was never recovered from viremic blood on EBK, OT, and BT cell cultures by immunofluorescence.

On the contrary, when mouse brain adapted BT 10 strain of the virus was inoculated on the cell cultures, fluorescing virus infected cells were consistently detected. Numerous large plaques of fluorescing cells were detected in the primary EOT cell cultures.

Limited attempts to adapt BT virus from sheep blood to cell culture by blind passages were not successful.

Effect of Freezing and Storage on Bluetongue Virus.—Bluetongue virus in sheep blood mixed with OPG was divided and a portion was maintained by 4° C. while the other was frozen at −78° C. for 48 hours. The two portions were each inoculated into susceptible sheep and the presence of viable BT virus was confirmed by diagnostic increases in CF titers from negative to 1-80 and 1-160 in both sheep.

To more precisely determine whether there was a loss of virus titer and to study the effect a storage of heparinized sheep blood containing BT virus at the temperature of an ordinary household freezer, a set of virus

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[2] Supplied by Dr. M. M. Jochim, Animal Disease Research Laboratory, ARS, USDA, Federal Center, Denver, Colo. 80225.

[3] OPG solution; potassium oxalate, 5 gm., phenol 5 gm., glycerin, 500 ml. and distilled water, 500 ml.

[4] Supplied by Dr. Paul Boulanger, Animal Pathology Division, Health of Animals Branch, Animal Diseases Research Institute, Hull, Quebec, Canada.

[5] Revco, Inc., Industrial Products Division, Deerfield, Mich.

aliquots were prepared and stored at −20° C. An initial titration of the virus employing one sheep per tenfold dilution was performed employing 1.0 ml., s.c. Virus was detected in the 10^{-2} but not in the 10^{-3} dilution.

A second titration of the virus blood was performed after 79 days of storage and virus was again detected in the 10^{-2} dilution.

The third and last trial was performed after the virus had been stored for 364 days at −20° C. Bluetongue virus was not detected in the 10^{-1} or 10^{-2} dilutions of the inoculum. The undiluted blood was not inoculated. A hundredfold reduction of virus titer had occurred during the storage in heparinized blood frozen at −20° C.

Bluetongue virus in heparinized sheep blood and mouse brain suspensions was stored at −78° C. without gross loss of potency. However, no precise titrations of BT virus stored in this manner were performed.

Discussion

The work of previous investigators was confirmed in that BT virus was propagated in lamb kidney cell cultures. However, the detection of the virus by immunofluorescence was not a consistent finding. In addition, other primary cell cultures and cell lines more readily available the year round did not grow the virus. It was concluded that the isolation of BT virus by cell culture from viremic sheep blood was not a reliable diagnostic technique. However, preliminary inoculation of the CE by the intravenous route or baby mice intracerebrally may be used to adapt the virus. A subpassage to a suitable cell culture may then be performed.

The immunofluorescent technique may be used to great advantage on the infected cell cultures since it has a broad spectrum reactivity and the conjugate will stain all strains of the virus.

Although the BT virus in sheep blood was not inactivated by freezing at −78° C. for 48 hours, the virus was adversely affected by storage at −20° C. for 1 year. Thomas (14) has recommended rapid freezing in an alcohol dry ice bath, storage at −78° C. and rapid thawing for the preservation of BT virus.

In view of the stability of the virus at ambient temperatures, the use of OPG solution for the collection of blood specimens for virus isolation is a useful procedure, particularly since intravenous inoculation of the CE remains the method of choice for initial propagation of the virus.

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BLUETONGUE VIRUS STUDIES: II. COMPLEMENT FIXING ANTIBODY RESPONSE IN EXPERIMENTALLY INFECTED CATTLE, SHEEP AND DEER. Carbrey, E. A., Gustafson, G. A. and Lee, L. R. (Project Report)

Summary

Two sheep and three cattle were infected with bluetongue (BT) virus and the complement-fixing antibody titers were determined at weekly intervals for 719 and 594 days, respectively. Peak titers of 1-80 and 1-160 were detected in both sheep and cattle at 40 to 50 days postinoculation (DPI). At the termination of the experiment the sheep had persistent titers of 1-40 while the titers of the cattle had fallen to 1-5 and 1-10.

Serums from deer infected with epizootic hemorrhagic disease (EHD) were tested against BT mouse brain antigen using the complement fixation test. The tests were negative indicating a lack of identity between BT and EHD virus. However, a consistent 20 percent fixation of complement was detected at the 1-5 dilution. This was considered evidence for a slight degree of antigenic relationship between the two viruses.

Introduction

The export of cattle and sheep to foreign countries provides an important source of income to livestock breeders. Other countries are concerned that these animals do not introduce new disease agents into their livestock populations. Bluetongue (BT) is a disease not found in many countries of the world, for instance, Canada, Great Britain, Australia, New Zealand and Brazil. Before our cattle and sheep are permitted to enter these countries, they must be certified as free of BT infection. The development of a reliable test for the detection of complement fixing antibodies against BT virus (1, 2, 3)[1] provided a serologic method for detecting previously infected animals. Some of the countries that were anxious to import our cattle and sheep agreed to accept those that were negative to the complement-fixation (CF) test.

Although the complement fixing antibody response to BT infection was well documented, information was not available on the duration of the titers detected or their relationship to a persistent viremia. This paper will describe experiments performed on experimentally infected sheep and cattle which were maintained in strict isolation for 719 and 594 days, respectively. During these periods the complement-fixing titers of these animals were determined at weekly intervals.

Serums from deer previously infected with BT and epizootic hemorrhagic disease (EHD)[2] were tested for complement fixing antibodies against BT to determine the degree of cross reaction produced by these closely related viruses.

Materials and Methods

Virus Strains.—Bluetongue virus strain California BT8, 10th sheep passage was obtained by bleeding an infected sheep 6 days postinoculation (DPI). It was supplied[3] in an anticoagulant preservative solution, OPG.[4] California (type 10) BT virus mouse brain passage was obtained as freeze dried mouse brain.[5]

Experimental Animals.—Sheep were obtained from two closed flocks located in Iowa that had been tested and found negative for BT by the CF test. All sheep were tested before inoculation and found negative.

Cattle were obtained from the laboratory herd that has been tested regularly and found free of BT infection.

All animals were maintained in strict isolation. Sheep used for virus isolation were maintained in plastic isolation cages provided with a negative air pressure.

Complement Fixation Test.—The test was performed according to the method of Boulanger, *et al.* as previously described. (2)

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[1] Numbers in parentheses refer to References at the end of this report.

[2] Supplied by Dr. F. C. Thomas, University of Wisconsin, Madison, Wis.

[3] Supplied by Dr. M. M. Jochim, Animal Disease Research Laboratory, ARS, USDA, Federal Center, Denver, Colo. 80225.

[4] OPG solution: potassium oxalate, 5 gm., phenol 5 gm., glycerin, 500 ml. and distilled water, 500 ml.

[5] Supplied by Dr. Paul Boulanger, Animal Pathology Division, Health of Animals Branch, Animal Diseases Research Institute, Hull, Quebec, Canada.

Experimental Procedure.—Two sheep and three calves were each inoculated i.m. with 4.0 ml. of 50 percent sheep blood in OPG and maintained in isolation rooms for the duration of the experiment. Rectal temperatures were determined during the acute phase of the disease at daily intervals. Serums for the CF test were collected at approximately weekly intervals. Blood samples for packed cell volume (PCB) and white blood cell determinations were obtained daily during the acute phase.

Following the acute phase the animals remained in good health except for one calf that was severely injured when it caught its foot under a gate. The sheep were maintained for 719 and the cattle for 594 days. At the termination of the experiment and at periodic intervals, blood was collected and inoculated into susceptible sheep to determine whether any of the animals had a persistent BT viremia. Usually 20 ml. of blood was collected; however, at the termination of the experiments 500 ml. of blood was obtained from each sheep and 1000 ml. from each calf. The test sheep were inoculated with the washed cells obtained from these samples.

Serums were obtained from deer experimentally infected with BT virus, vaccine strain. Other deer were given two inoculations of EHD virus subcutaneously with an interval of 2 weeks between inoculations. Serums were collected 2 weeks after the second inoculation (4).

Results

Sheep.—Following exposure to BT virus the two sheep each had a marked increase in rectal temperatures to 41.2° C. (106.0° F.) and 40.6° C. (105.0° F.) on DPI, four and six. The only additional clinical sign detected was edema of the lips. Complement fixation titers of 1-5 and 1-20 were first detected 20 DPI and titers of 1-80 and 1-160 were attained at 40 DPI. The titers gradually receded but remained well above the critical diagnostic level of 1-20 through 461 DPI. At this time 20 ml. of blood were obtained from each sheep and inoculated into susceptible sheep to check for the presence of BT virus. The inoculated sheep remained negative to the CF test. At the termination of the experiment, at 719 DPI, the sheep had titers of 1-40. Large blood samples of 500 ml. were collected and the cellular elements were inoculated into susceptible sheep. Serologic evidence of BT infection was not detected.

Three Holstein calves, two heifers and a bull, were inoculated with BT virus. A severe leukopenia and slight pyrexia was detected at 5 DPI and a second period of illness was observed from 10 - 13 DPI. At 13 DPI serum obtained from the calves was negative by CF test; however, by 20 DPI two of the calves had titer, 1-5 and 1-10. By 55 DPI titers of 1-80 and 1-160 were found and the titers persisted at this level through 150 DPI (fig. 1). At this point the titers began to decrease and by 319 DPI had fallen to 1-10 in two of the animals and 1-40 in the third. Eventually at 468 DPI the titers dropped below the minimum designated positive titer of 1-20 and remained in the suspicious level of 1-5 and 1-10 through the termination of the experiment at 594 DPI.

Attempts were made to recover BT virus from the blood of the calves at 320 and 550 DPI. At 320 DPI 20 ml. of blood from each calf was inoculated into a susceptible sheep and at 550 DPI the cellular elements of 1000 ml. of blood were inoculated. The inoculated sheep remained healthy and failed to develop any detectable titers to the CF test.

The rate of change in the mean titers (slope) was calculated and found to be a log titer decrease per day of 0.0021684 (fig. 1). This corresponded to approximately one dilution decrease in CF titer every 6 months.

The standard error of the CF test as measured by the application of analysis of variance of the data was a log titer of 0.4364 or slightly more than a twofold dilution. With a system of statistical analysis in which dummy variables were employed, significant differences were found in the overall antibody response of two of the three calves.

The CF test was performed on serums collected from deer before and after experimental infection with either BT or EHD. The serums from the BT infected deer had titers of 1-20 while the serums from the EHD infected deer were negative at the 1-5 dilution (table 1).

A minimal fixation of complement, approximately 20 percent, was consistently detected in the 1-5 dilutions of the serums from the EHD infected deer.

This titration was performed as a blind study using coded serums.
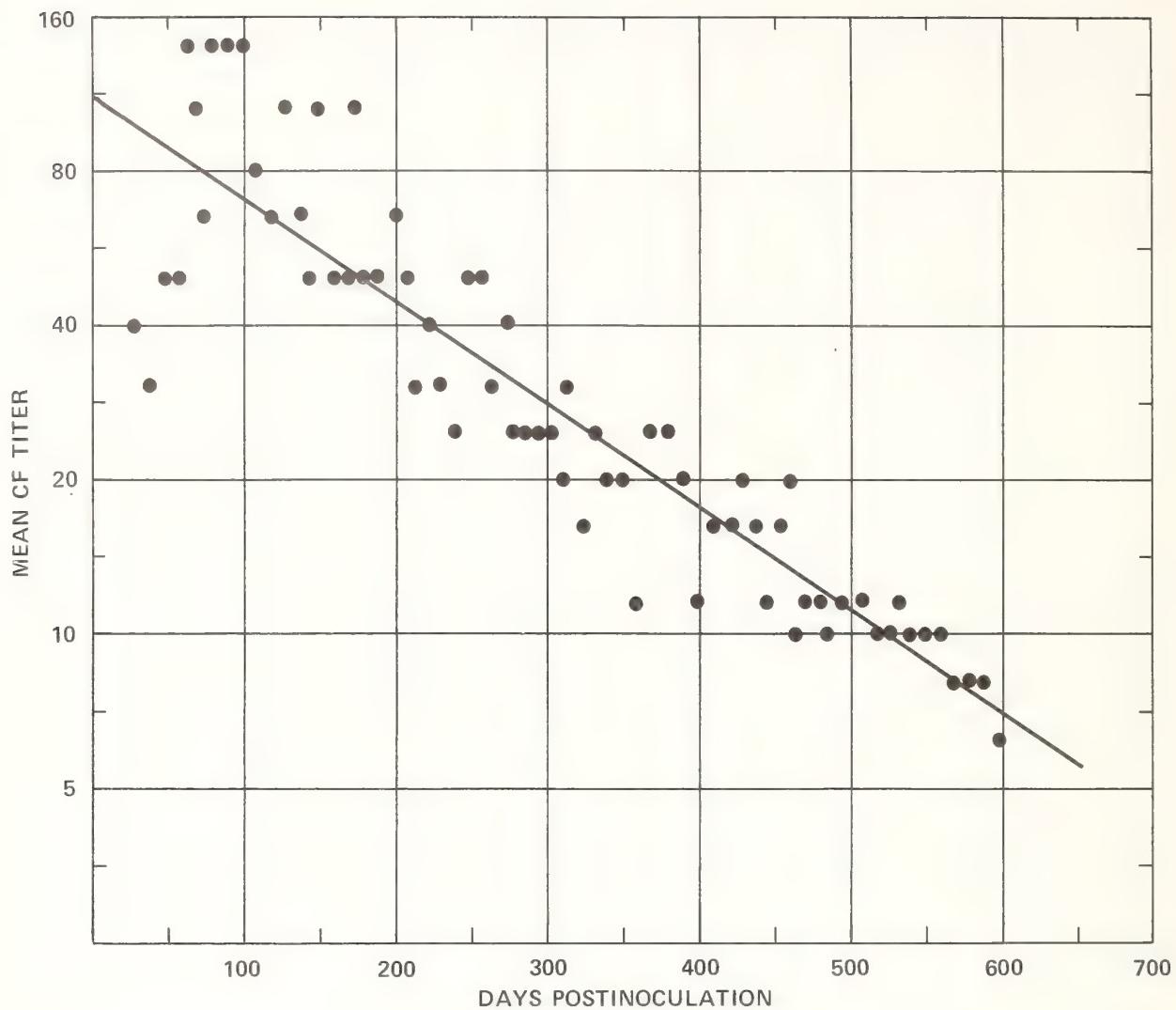
Figure 1.—Computer plot of the mean complement fixation titers and their slope of three calves experimentally infected with bluetongue virus.

Table 1.—Complement fixation titers of deer experimentally infected with Bluetongue or Epizootic Hemorrhagic disease

Serum no.	History	CF titer	Classification
1	Pool (6 serums) Pre. Inoc.	N[1]	Negative
2	Pool (6 serums) Post Inoc., 3-4 Weeks Bluetongue	20	Positive
3	Serum, 3-4 Months Post Inoc., Bluetongue	20	Do.
4	(1 serum), 3-4 Months, Post Inoc., Bluetongue	20	Do.
5	(1 serum), Pre. Inoc.	N	Negative
6	(1 serum). Pre. Inoc.	N	Do.
7	(1 serum), Pre. Inoc.	N	Do.
8	(1 serum), Post Inoc., 1 Month, EHD	N[2]	Do.
9	(1 serum), Post Inoc., 1 Month, EHD	N[2]	Do.
10	(1 serum), Post Contact, 1 Month, EHD	N[2]	Do.

[1] Less than 50 percent fix at 1-5 dilution.
[2] 20 percent fix at 1-5 dilution.

Discussion

The intensity and duration of the complement fixing antibody response was marked in both sheep and cattle and may have been related to the virulence of the BT8 strain of the virus. However, the presence of a viremia was not detected during or at the termination of the experiment. The sensitivity of the CF test in detecting cattle and sheep previously infected with BT was demonstrated.

The failure of the serums from EHD infected deer to fix complement with BT antigen except at a barely detectable level was of great significance. The EHD virus is similar in physical and chemical characteristics to BT and some degree of cross reaction might have been expected. All strains of BT virus will fix complement with a mouse brain antigen prepared from any strain of the virus. If the EHD virus was just another strain of BT virus, a strong reaction would have been detected between the BT mouse brain antigen and the serums from the EHD infected deer. This serological data is evidence for the uniqueness of EHD as a distinct and separate agent from BT virus.

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BOTULISM

DIAGNOSIS OF PHEASANT BOTULISM, Proctor, S. J., Cassidy, D. R. and Harrington, R. (Project Report)

Summary

Botulism in pheasants caused by the ingestion of fly maggots containing type C botulinum toxin was diagnosed. Diagnosis was based upon finding the source of toxin and demonstrating its presence in serum of acutely intoxicated pheasants.

Introduction

Animal botulism is initiated when feed containing botulinum toxin is ingested. Clostridial spores usually pass through the digestive tract without harming animals. Culturing *Clostridium botulinum* from gastrointestinal contents does not constitute a basis for the diagnosis of botulism. Demonstration of botulinum toxins in animal feed or intestinal contents is a better index of intoxication, but a definitive diagnosis depends upon the demonstration of botulinum toxins in serum or tissue samples.

Pheasant botulism on game bird farms presents some unique features. Environmental conditions such as crowding, dense cover in pens, fly larvae, ubiquity of *C. botulinum* spores, weather conditions, and the pheasants' avid seeking of larvae and worms all contribute to botulism epornitics on game farms. Pheasants are susceptible to botulinum toxins types A, C, and E. They are relatively resistant to type B (3, 6).[1]

The following report concerns the diagnosis of botulism on a game bird farm.

Case Report

In 1969 and 1970, from August to September, severe losses were experienced in a group of 7,000 Chinese ring-necked pheasants, *Phasianus colchicus torquatus*, on a game bird farm in Kansas. Progressive ataxia, weakness, "walking" on wings and flaccid paralysis were noted in the pheasants. No significant gross or microscopic lesions were observed.

Severe losses were again experienced in September 1971, on the same game farm. Clinical signs were progressive flaccid paralysis and acute death. Affected pheasants were found lying on their sides or sternums. A dense growth of grass, milo, and weeds, which hid dead bird carcasses, was present in each pen. Automatic waterers and turkey feeders were present in each pen; however, feeder space was not adequate.

Several fly-blown pheasant carcasses were collected from the pens, placed on dry ice, and transported to the laboratory. Blood was collected from 10 acutely sick birds to obtain serum which was then frozen and transported to the laboratory. Necropsies were performed on the sick birds and tissues collected for histologic examination. Increased pericardial fluid was the only consistent necropsy lesion.

Laboratory Procedures and Results

Fifty blowfly larvae from two pheasant carcasses were macerated with a mortar and pestle and suspended in 50 ml. of phosphate-buffered saline which was centrifuged at 5000 r.p.m. for 5 minutes. Mouse neutralization tests were performed on the supernatant and 10 pheasant serums (1). Type C botulinum toxin was found in the supernatant and serum. Type C botulinum antitoxin or heat neutralized the lethal toxin.

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[1]Numbers in parentheses refer to References at the end of this report.

Discussion

Pheasant botulism differs clinically from chicken botulism. Loose feathers and prolapse of the third eyelid observed in chickens are not clinical features of pheasant botulism. Flaccid paralysis is the predominant clinical sign. Two facets of pheasant botulism may be used to make a diagnosis. First, a source of botulinum toxin must be established. Dead fly-blown pheasant carcasses have been reported to be the predominant source of toxin (6). Spores of *C. botulinum* are swallowed while pheasants are picking up grit. Subsequent unrelated deaths may initiate an outbreak of botulism under proper environmental conditions. Anaerobic conditions exist in dead birds facilitating *C. botulinum* growth and toxin production. Maggots aid in maintaining a suitable pH in the carcass allowing continued growth and toxin production. Botulinum toxin does not affect maggots (8). Maggots are thought to concentrate botulinum toxins (6), but this has not been proven experimentally. Pheasants apparently find fly-blown carcasses in the weeds and ingest the larvae. The presence of *C. botulinum* and its toxin in the carcasses can be determined by culturing the organism or by macerating the larvae and injecting mice.

The second and most difficult step in the diagnosis of pheasant botulism is finding the toxin in serum or parenchymatous organs of affected birds. Several pheasants may be needed to demonstrate the presence of toxin in serum. Blood samples should be obtained from birds as soon as possible after clinical signs are noted. Serum toxin titers apparently decline rapidly, frequently preventing a laboratory diagnosis. The decline in serum toxin titers in experimental animals is not due to toxin excretion (7). In rats botulinum toxins leave the serum and are so firmly bound to neuromuscular junctions that antitoxin will not remove them (4). During reinnervation the nerve sprouts and grows around the old neuromuscular junction (2). This firm neuromuscular binding has also been reported to account for the accumulation of toxin in mink and mice (5). Undetectable levels of toxin in the mink feed have been shown experimentally to cause clinical botulism without detectable serum levels (5). Therefore, failure to find toxin in serum may indicate the titers are too low to detect (since botulism is a cumulative toxin) or the toxin has disappeared from serum prior to collection. Several attempts may be necessary to make a definitive diagnosis.

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BRUCELLOSIS

In order to attain established goals of the Brucellosis Eradication Program, it is necessary to substantially increase surveillance testing of market cattle and swine. Many brucellosis testing laboratories are already being utilized to full capacity, therefore, a rapid inexpensive screening test is necessary. A screening test for brucellosis, employing microtiter equipment and techniques, is described and evaluated.

Columbia, Targhee, and Rambouillet rams were artificially exposed to *Brucella ovis* via the conjuctival sac. Rams not shedding *B. ovis* in their semen were re-exposed intravenously 13 weeks, and again 27 weeks, after the initial exposure.

Ewes, induced to come in estrus, were mated with rams that were shedding *B. ovis* in their semen. Subsequently, during the same period of estrus, each ewe was mated with two non-infected vaccinated or non-vaccinated rams. After mating, the infected rams were placed in a pen with vaccinated and non-vaccinated rams. These rams were allowed to cohabit for approximately 1 year.

Serologic responses occurred in all 48 artificially exposed rams, in 8 of 25 venereal contact non-vaccinated rams, and in 5 of 23 cohabitation contact non-vaccinated rams. The serologic responses of the venereal and cohabitation contact vaccinated rams were difficult to evaluate because of persistent vaccinal titers.

Brucella ovis was isolated from the semen of 14 of 48 artificially exposed rams. Single colony isolations were made from the semen of each of two cohabitation contact vaccinated rams on one occasion. There was no other cultural evidence of transmission of infection by venereal or cohabitation contact.

ENCEPHALOMYELITIS

Eastern encephalomyelitis virus (EEV) was isolated in cell culture from the brain of a 3-week-old pig that had signs of central nervous system (CNS) disturbance. The pig was from a herd of 30 sows and 200 pigs in southern Georgia. More than 160 of these pigs died during the months of November and December. Swine pox, inclusion body rhinitis, strongyloidosis, salmonellosis, and erysipelas were also demonstrated in the herd.

Four of the nine nursing pigs (in a litter of 11) inoculated with either the EEV isolant in cell culture fluid or 10 percent brain suspension developed CNS signs similar to those seen in the naturally infected pig. Brain lesions in these pigs were characterized by neutrophils in perivascular cuffs and in necrotic areas. Neither the sow nor any of the remaining pigs developed clinical illness. Five pigs without clinical evidence of encephalitis developed serum-neutralizing antibodies against EEV. The sow and two pigs exposed by contact did not have detectable antibodies at the end of 14 days.

EQUINE INFECTIOUS ANEMIA

EQUINE INFECTIOUS ANEMIA IMMUNODIFFUSION TEST LABORATORY SURVEY. Pearson, James E.

Diagnostic laboratories approved by APHIS, USDA, to perform the equine infectious anemia (EIA) immunodiffusion (ID) test are required to test a set of unknown check test serums at periodic intervals. The purpose of this requirement is to insure that adequate quality control standards are maintained throughout the Nation and a reasonable degree of uniformity in test results may be expected by the horse industry. The check list samples also provided a form of training as the serums are more difficult than those routinely encountered.

Check Samples

Fifty samples were determined to be optimum for this evaluation. This number is considered statistically significant in a test where the finding is either positive or negative. A smaller number of samples would be required in a test where antibody titers are determined.

Forty-eight laboratories participated in the survey.

Samples selected contained enough serums so that each laboratory received identical 0.5 ml. amounts. This permitted each sample to be tested at least three times. Some of the samples selected were the most difficult that were available. It was anticipated that few laboratories would get 100 percent.

The samples were classified in five categories: negative—13; very weak positive—2; weak positive—14; positive—16; and strong positive—5. Several of the negative samples formed lines of nonidentity with certain antigens. Samples were obtained from a variety of sources. Several were from horses hyperimmunized with other antigens that contained equine serum and, as a result, produced lines between the serum wells. Other samples were from experimental horses that had been infected with EIA for various lengths of time. The remainder of the samples were interesting samples we had received for routine testing. A few of the samples had been diluted or mixed to obtain the desired reaction.

Several of the samples were of very poor quality with a high lipid content. If these had been submitted for routine diagnostic purposes another sample should have been requested. However to provide a standard comparison, results for all samples were required.

All samples were tested at least 10 times with at least five different antigens before being used as check samples. Personnel that had received training at Veterinary Services Diagnostic Laboratory, Ames, Iowa, since June 1972 had tested at least half of the samples as part of their training.

Evaluation of Results

Each laboratory received their test score and a drawing of the reactions that were observed when the samples were tested. The average number of serums scored incorrectly was 6.7. When the results of all the laboratories were plotted the data was found to approximate a Poisson distribution. The laboratories that were wrong on more than 10 serums fell below the 95 percent confidence limit and were considered unsatisfactory.

Those laboratories that fell below the 95 percent confidence interval will receive special training, including practice samples similar to those that they missed. Every one will receive another group of check samples which must be tested with an improved score if the laboratory is to remain approved.

Three of the 48 laboratories scored correctly on all of the samples.

Discussion

Several potential sources of error in the test were revealed by the check tests that were not covered adequately in the protocol (1).[1,2] There is an optimum amount of moisture that the plates should contain when they are used. If there is an excess of moisture, particularly if it is visible in the wells, the plate must be dried before use. Therefore, the following change is recommended in the protocol. After the 1-percent layer is poured the plates should be allowed to dry 1 to 2 hours with the lids off at room humidity before the reagents are added to the wells. It is still recommended that a moist chamber be used for incubation, particularly in the dryer climates. It has been noted that if the plates become too dry the lines are weaker so the plates should not be allowed to set for long periods before being used. Each laboratory will probably have to adjust its method a little to compensate for the humidity in the laboratory room. The amount of moisture can be gauged by the speed with which the reagents evaporate. Also if the sides of the wells do not remain perpendicular after being cut there is an excess of moisture in the agar.

Care must be taken to insure that the wells are completely filled. To insure getting adequate serum in the well, it should be filled level with the agar surface without leaving any meniscus.

Some laboratories did not detect some of the strong positive samples. If the control line stops at some distance from the unknown well the sample must either be a strong positive or it is an unsatisfactory test and should be repeated. If the control line is clearly visible and goes into an adjacent negative serum well the unknown sample must be a strong positive even though there is no line of identity (fig. 1). This finding can be confirmed by making doubling dilutions of the unknown serum. A line of identity should appear with the diluted serum.
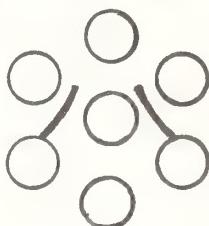
Figure 1

The weak positive serums were missed most frequently. Any plate not containing a good control line should be discarded and the test repeated. With many of the check serums that were very weak the control precipitin lines only hooked at the edge of the test serum well. If the control line is weak and does not extend to the well the weak positive sample cannot be detected. It has been our experience that these serums are the ones that cause conflicting results between laboratories. This does affect confidence in the test, even though these horses may be of doubtful epizootiological importance.

In a few cases, a sample that produced a strong line of non-identity was called positive. This is another case where a strong control line is required. The angle formed between a line of non-identity and the positive control line is different than that formed by a line of identity (fig. 2).
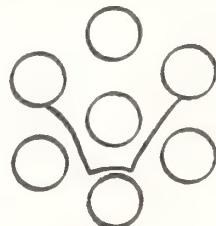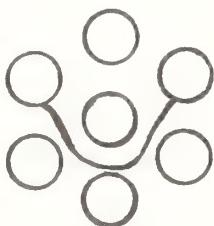
Figure 2

[1] Numbers in parentheses refer to References at the end of this report.

[2] "Protocol for the Immunodiffusion (Coggins) Test for Equine Infectious Anemia." Prepared by James E. Pearson, DVM, in collaboration with the Committee on Equine Infectious Anemia of the American Association of Veterinary Laboratory Diagnosticians, E. A. Carbrey, Chairman, D. Barnett, L. Coggins, J. R. Gorham, W. L. Kadel, W. W. Kirkham, J. E. Pearson, E. Roth (October, 1971).

We have found that it will sometimes take at least 72 hours before the reaction is complete enough to make a reading. Therefore, 72 hours should be allowed even though it is often possible to report results in 48 hours.

It has been noted that unreliable reactions are produced if the lyophilized antigen does not retain a vacuum during storage. Therefore any vial not containing a vacuum should be returned to the company.

The results of laboratories participating in the check testing program were generally very good. This replication of test results between laboratories will insure that the EIA ID test will continue to be regarded as an accurate diagnostic technique.

ERYSIPELAS

Six specific-pathogen-free (SPF) pigs were exposed to *Erysipelothrix rhusiopathiae* by a slap tattoo. One of the exposed pigs developed acute swine erysipelas. This indicates that swine erysipelas can be transmitted by the slap tattoo.

SALMONELLA AND ERYSIPELOTHRIX INFECTION IN SWINE SUSPECTED OF HAVING HOG CHOLERA.
Harrington, R. and Hulse, D. C. (Project Report)

Summary

Frequently the diagnosis of hog cholera is complicated by clinical signs which in many ways are similar to those seen in septicemic disease caused by bacteria. Although there are several bacterial diseases that may cause clinical signs somewhat similar to hog cholera, acute swine erysipelas and acute salmonellosis are the two most frequent bacterial diseases that may produce clinical signs indistinguishable from hog cholera. Bacteriologic examination should be performed for differential diagnosis of these diseases.

A major responsibility of the General Bacteriology Unit of the Diagnostic Bacteriology Section, VSDL, has been the bacteriologic examination of swine tissues from hog cholera negative specimens. Results from these examinations have provided valuable information for differential diagnosis of hog cholera in support of the hog cholera eradication program.

This report summarizes a study of the occurrence of salmonella and erysipelothrix in tissues of swine from cases where hog cholera was suspected during calendar years 1971 and 1972.

Materials and Methods

Tissues were obtained from swine in herds where hog cholera was suspected by State and Federal regulatory veterinarians. The tissues were submitted to the General Bacteriology Unit following examination for hog cholera virus by the Swine Viruses Unit of the Diagnostic Virology Section, VSDL. Approximately 95 percent of the tissues were spleens.

Culture methods for the isolation of erysipelothrix and the fluorescent antibody technique for the detection of salmonellae have been described (1, 3).[1]

Results and Discussion

Bacteriologic examinations were conducted on 4,273 swine tissues (table 1). This represents 3,138 animals from 2,106 herds in 43 States and Puerto Rico. Salmonellae were detected in one or more tissues from 37.6 percent of the animals. *Erysipelothrix rhusiopathiae* was isolated from one or more tissues from 21.4 percent of the animals. *Salmonellae* and *E. rhusiopathiae* were detected in one or more tissues from 8.7 percent of the animals.

The number of specimens examined from many of the States was not large enough to make analysis of the results meaningful. Nevertheless, a breakdown of the salmonella and erysipelothrix results by State are presented in table 1. The States with the highest percentage of salmonella positives, from 100 or more animals, were Indiana (56.3 percent), Ohio (45.1 percent), Louisiana (27.6 percent), Oklahoma (26.9 percent) and Minnesota (23.8 percent). The States with the highest percentage of erysipelothrix positives, from 100 or more animals, were Oklahoma (30.8 percent), Louisiana (28.6 percent), Texas (26.6 percent), Indiana (18.2 percent), Ohio (13.3 percent) and Minnesota (10.3 percent).

The results of this study do not depict the true incidence of salmonella and erysipelothrix infections in swine. Only on rare occasions was the laboratory called upon to perform bacteriologic examinations on tissues from swine other than those suspected of having hog cholera. Nevertheless, as previously reported (2, 4), the data indicate that a large segment of the swine population in the States sampled is infected with salmonella and erysipelothrix. Since

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[1] Numbers in parentheses refer to References at the end of this report.

Table 1.—Salmonella[1] and Erysipelothrix results (by State) from 4,273 tissues obtained from swine suspected of having hog cholera

State	No. of Animals	No. of Herds	Salmonella positive	Salmonella positive	Erysipe- lothrix positive	Erysipe- lothrix positive	Salmonella and Erysipe- lothrix positive	Salmonella and Erysipe- lothrix positive
				Percent		Percent		Percent
Arizona	7	5	2	28.6	4	57.1	1	14.3
Arkansas.	95	78	29	30.5	20	21.0	4	4.2
California	1	1	1	100.0	1	100.0	1	100.0
Colorado	22	17	5	22.7	9	40.9	2	9.1
Connecticut	4	4	0	0	3	75.0	0	0
Delaware	2	2	2	100.0	0	0	0	0
Florida	2	2	0	0	1	50.0	0	0
Georgia.	53	43	21	39.7	17	32.0	5	9.4
Idaho	1	1	0	0	1	100.0	0	0
Illinois	14	10	3	21.4	1	7.1	1	7.1
Indiana.	695	403	391	56.3	127	18.2	67	9.6
Iowa	45	28	16	35.6	6	13.3	3	6.7
Kansas	6	5	2	33.3	2	33.3	2	33.3
Kentucky	19	14	11	57.9	3	15.0	0	0
Louisiana	105	86	29	27.6	30	28.6	11	10.5
Maryland	13	8	6	46.2	4	30.8	4	30.8
Massachusetts	15	5	5	33.3	1	6.7	1	6.7
Michigan.	3	3	2	66.7	1	33.3	1	33.3
Minnesota.	340	182	81	23.8	35	10.3	13	3.8
Mississippi	4	2	0	0	3	75.0	0	0
Missouri	27	2	1	3.7	2	7.4	1	3.70
Montana.	2	2	2	100.0	1	50.0	1	50.0
Nebraska	22	15	13	59.1	8	36.3	3	13.6
New Jersey.	66	48	38	57.6	12	18.2	8	12.2
New Mexico	8	8	1	12.5	1	12.5	1	12.5
New York	12	10	5	41.7	2	16.7	1	8.3
N. Carolina.	50	28	25	50.0	8	16.0	6	12.0
N. Dakota	11	8	5	45.5	0	0	0	0
Ohio.	226	150	102	45.1	30	13.3	14	6.2
Oklahoma	156	117	42	26.9	48	30.8	17	10.9
Pennsylvania	6	3	1	16.6	0	0	0	0
Puerto Rico	30	21	8	26.6	6	20.0	2	6.7
Rhode Island	1	1	0	0	0	0	0	0
S. Carolina	25	12	6	24.0	5	20.0	2	8.0
S. Dakota	1	1	0	0	0	0	0	0
Tennessee.	5	3	2	40.0	0	0	0	0
Texas	998	745	307	30.7	266	26.7	96	9.6
Utah	11	8	6	54.6	3	27.3	3	27.3
Vermont.	2	2	0	0	0	0	0	0
Virginia.	15	7	4	26.7	4	26.7	0	0

See footnote at end of table.

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Table 1.—Salmonella[1] and Erysipelothrix results (by State) from 4,273 tissues obtained from swine suspected of having hog cholera—Continued

State	No. of Animals	No. of Herds	Salmonella positive	Salmonella positive	Erysipelothrix positive	Erysipelothrix positive	Salmonella and Erysipelothrix positive	Salmonella and Erysipelothrix positive
				Percent		Percent		Percent
Washington	7	6	1	14.3	3	42.9	0	0
W. Virginia	9	7	3	33.3	3	33.3	2	22.2
Wisconsin	1	1	1	100.0	0	0	0	0
Wyoming	3	1	0	0	0	0	0	0
TOTAL	3,140	2,105	1,179	37.6	670	21.3	273	8.7

[1] FA technique was used for detection of salmonella.

these organisms are capable of producing complicating diseases with clinical signs indistinguishable from hog cholera, it is of epidemiological importance to conduct bacteriologic examinations on swine tissues when hog cholera is suspected.

Acknowledgment

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Susceptible sows in various stages of pregnancy were vaccinated simultaneously with attenuated or virulent (Ames) hog cholera (HC) virus and anti-HC serum. Tissue specimens from offspring of the sows were examined for HC virus by the fluorescent antibody cell culture technique (FACCT). Isolates from the newborn pigs and composite suspensions of tissues that gave negative results by the FACCT were inoculated in susceptible pigs for confirmation.

Transplacental infection occurred in four sows vaccinated with Ames virus and either 35 or 75 ml. of anti-HC serum. The dose of antiserum and stages of gestation in which the vaccination was performed were factors that were considered to affect the results of the experiment. These four sows were in the first month of pregnancy and three of the four sows had been given the smaller dose (35 ml.) of antiserum.

Transplacental infection was not confirmed in 12 sows vaccinated with attenuated virus and 20 ml. of anti-HC serum nor in 8 sows vaccinated with Ames virus and either 35 or 75 ml. of antiserum.

MYCOBACTERIOSIS

A COMPARISON OF HISTOPATHOLOGIC TECHNIQUES FOR DETECTING ACID-FAST BACILLI, Mote, R. F., Gigstad, D. C., Himes, E. M. and Muhm, R. L. (Project Report)

Summary

Two hundred and fifty suspected tuberculous bovine specimens were examined for acid-fast bacilli using three different histopathologic techniques. These included an auramine o-stained smear, an auramine-o stained tissue section and a new fuchsin-stained tissue section procedure. The combination of an auramine o-stained smear and an auramine o-stained tissue section had a statistically significant advantage for detecting mycobacteria.

Introduction

In support of the bovine tuberculosis eradication program, suspected granulomas received from slaughter establishments are routinely examined at Veterinary Services Diagnostic Laboratory, Ames, Iowa. Upon collection, tissues are split and one portion is put in 10 percent buffered formalin to be examined by the Histopathology Section and the other portion is put in saturated sodium borate solution for examination by the Mycobacteriology Section. This study presents histopathology results from 250 suspected tuberculous submissions and compares three different staining procedures. Also included are mycobacteriology results if available.

Materials and Methods

Histopathologic preparations for microscopic examination were made on each of 250 submission suspected of being tuberculous. These were "run of the mill" specimens, biased only by selection for the presence of a gross lesion large enough to furnish sufficient material for examination. There was some variation in the content of the individual submissions. Some contained only lymph node, others lymph nodes and lung, or liver, or spleen or various other combinations of tissues.

Tissues were removed from formalin, examined for lesions, cut in for paraffin impregnation and a portion of lesion was collected for smear preparation. Smears were stained with auramine-o (AO) (2).[1] Paraffin-impregnated tissues were blocked, sectioned at six microns and mounted on slides. Replicate tissue sections were prepared, one stained with a modified new fuchsin (NF) (1) procedure, another stained with a AO preparation (3) and a third stained with hematoxylin and eosin. A Leitz Orthoplan microscope equipped with a Xenon lamp and BG-12 blue light, BG-38 heat and K-530 barrier filters was used for fluorescent examinations. A Leitz Orthoplan equipped for light microscopy was used for examination of H&E and NF-stained sections.

Mycobacterial isolation and typing results were obtained for 220 of the 250 submissions. (Some had not been submitted for culture and others not examined because they had been previously diagnosed as Coccidioidomycosis.)

A comparison of the techniques for detecting acid-fast bacilli was made to determine if there were statistically significant differences in efficiency using one or a combination of staining procedures.

Results

The results of using the different techniques to detect acid-fast bacilli have been presented in table 1. The AO-stained smear and AO-stained section combination had a significant advantage when compared statistically with a combination of the AO-stained smear and the NF-stained section (p. >.05). More positives were detected in

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[1] Numbers in parentheses refer to References at the end of this report.

Table 1.—Comparison of diagnostic methods on 250 suspected tuberculous submissions

AO smear		AO tissue section	NF tissue section	Histopathology cases	Mycobacterial culture[1]
+		+	+	68	[2] 51/67
+		+	-	48	35/47
+		-	+	1	1/1
-		+	+	5	2/5
+		-	-	15	11/14
-		+	-	6	3/6
-		-	+	0	0/0
-		-	-	107	11/80
Total	132+ 118-	127+ 123-	74+ 176-	250	114/220

+ Acid-fast bacilli demonstrated.

- No acid-fast bacilli demonstrated.

[1] Some specimens were not cultured from mycobacteria (not received or previously diagnosed by histopathology procedures as Coccidioidomycosis).

[2] Number of isolations/number of specimens cultured.

AO-stained smears than in AO-stained sections but the difference was not significant (p. <.01). Mycobacteria were cultured from 114 of 220 specimens examined. Types isolated included *M. bovis* (43), *M. avium* (49), *M. paratuberculosis* (1), Runyan's Group III (13) and Runyan's Group IV (5).

Discussion

Heavily encapsulated, extensively mineralized lesions from tuberculous cattle often contain few detectable acid-fast organisms. This seems to be especially true in an *M. bovis* infection and trying to find the bacillus in such tissue sections can be a laborious, time-consuming process. This study was designed to determine if any procedure, or combination of procedures, might be superior to conventional fuchsin staining for demonstrating mycobacteria.

The AO-stained smear and the AO-stained tissue section combination was superior in that acid-fast bacilli were found in all specimens that were positive by any of the three staining procedures. The AO-stained smear and the NF-stained tissue section failed to detect acid-fast organisms in six specimens that were positive on AO-stained section.

In general, fluorescent microscopy methods using AO-stained preparations were easier to interpret and took less time than using light microscopy for NF-stained sections. In addition, the variety of types isolated indicate that the AO-staining procedures are an efficient method for demonstrating mycobacteria.

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This study was done to compare the serum lipid-lipoprotein profiles of tuberculin-positive and tuberculin-negative monkeys from the same colony in which an outbreak of tuberculosis had occurred.

In 21 tuberculin-negative monkeys, a beta lipoprotein component was present in the serum lipoprotein profile, which was not detected in the sera of five tuberculin-positive monkeys. Serum triglyceride values were significantly larger ($P < 0.001$) in the tuberculin-positive monkeys than in the tuberculin-negative animals. No differences were observed in serum cholesterol or in serum phospholipid-phosphorus values of the two groups of monkeys.

The fatty acid methyl esters extracted from cells of 10 strains of *Mycobacterium marinum* were analyzed by gas-liquid chromatographic analysis. The fatty acid profiles of each of the strains included saturated fatty acids ranging from C_8 to C_{24} plus some unsaturated analogues and a fatty acid tentatively identified as tuberculosteric acid. A comparison of the cellular fatty acid profiles of strains of *M. marinum* and of 35 strains of *M. kansasii* revealed similarities within each species but differences between these two photochromogenic mycobacteria. A branched-chain fatty acid characteristic of *M. kansasii* was found in only trace amounts in two of the 10 strains of *M. marinum*.

EFFECT OF STREPTOMYCIN THERAPY ON SERUM LIPID-LIPOPROTEIN PROFILES OF RABBITS INFECTED WITH MYCOBACTERIUM BOVIS. Thoen, C. O., Karlson, A. G., and Ellefson, R. D. The Journal of Infectious Diseases, Vol. 127, April, 1973, pp. 408-414. (Abstract of Published Report)

Experiments were done to determine the effect of appropriate antibiotic therapy on the increased lipids and lipoproteins in serum during infection of rabbits with *Mycobacterium bovis*. This organism was selected because it produces progressive disease consistently in rabbits and because the disease can be satisfactorily treated with streptomycin.

Lipid-lipoprotein analyses were made on whole serum and on ultracentrifuge fractions of serum by electrophoresis on paper strips presoaked in albumin-barbitol buffer solution. Serum cholesterol, phospholipid-phosphorus and triglyceride analyses were made using colorimetric tests.

In rabbits infected intravenously with *Mycobacterium bovis* the serum lipid levels were markedly elevated in 17 days. Lipoprotein analysis revealed dramatic increases of very low density lipoproteins associated with concomitant decreases of high density and of low density components. In untreated rabbits these changes persisted until death. In animals treated with streptomycin starting 17 days after infection, the concentrations of lipids and lipoproteins in serum returned to the pre-infection levels by day 30 of therapy.

EPIDEMIOLOGY OF INFECTIONS WITH SEROTYPES OF MYCOBACTERIUM AVIUM COMPLEX IN SWINE AND OTHER SPECIES. Thoen, C. O., Karlson, A. G., and Ranney, A. F. Proc. of the 76th Ann. Mtg. of the USAHA, 1972, pp. 423-436. (Abstract of Published Report)

Mycobacterium avium and closely related mycobacteria have been isolated from many species of animals and from man. These closely related acid-fast bacilli, referred to as *M. avium* complex bacteria, have a wider range of hosts than any other mycobacteria. A system for designating serotypes of *M. avium* complex bacteria is described and information is presented on the occurrence of infections with the different serotypes of *M. avium* complex in swine, chickens, cattle and man. A review is made of what is known and what is unknown about these mycobacterial infections in swine and other species.

AN OUTLINE OF METHODS FOR THE ISOLATION AND IDENTIFICATION OF MYCOBACTERIUM AVIUM COMPLEX ORGANISMS FROM SWINE TISSUES. Thoen, C. O., Richards, W. D., and Jarnagin, J. L. Proc. of the 76th Ann. Mtg. of the USAHA, Nov., 1972, pp. 440-443. (Abstract of Published Report)

An increased interest in *Mycobacterium avium* complex bacteria by laboratory diagnosticians resulted from changes in Meat and Poultry Inspection Program regulations of the United States Department of Agriculture which required more restrictive measures for disposition of animals with tuberculous lesions. In this report some of the laboratory methods used for isolating acid-fast organisms from swine tissues and the tests available for identifying strains of *M. avium* complex are outlined. The different solid and liquid media useful in culturing mycobacteria are described.

THERMOGRAPHY OF TUBERCULIN REACTIONS IN CATTLE. Merkal, R. S., Larsen, A. B., Nelson, H. A. and Pier, A. C. Infection and Immunity, Vol. 7, No. 5, May, 1973, pp. 805-808. (Abstract of Published Report)

The allergic reactions of dermal induration and hyperthermia were compared in tuberculin-hypersensitive calves. Tuberculins prepared from *Mycobacterium avium*, *M. bovis*, and *M. paratuberculosis* were administered intradermally and the responses were compared. The indurations from immediate and delayed hypersensitivity reactions were readily distinguishable, but the hyperthermic responses appeared to contain elements of both immediate and delayed hypersensitivity.

HISTOPATHOLOGIC EXAMINATION OF SUSPECTED TUBERCULOUS SUBMISSIONS, VSDL, FY73.

Muhm, R. L., Mote, R. F., and Himes, E. M.

During FY 1973, field submissions totaling 2,433, were examined by the Diagnostic Histopathology Section, VSDL, for lesions of tuberculosis. Nine hundred and eighty-two of these, approximately 40 percent, were determined by microscopic examination to contain granulomatous lesions with acid-fast bacilli and were reported as "compatible for mycobacteriosis." Seventeen percent were "suggestive of tuberculosis," there were "no significant findings" in 11 percent (mostly from NGL reactors or suspects) and 22 percent as "other" including Actinomycosis, Coccidioidomycosis, neoplasms, and parasites. Fifty-seven percent of the submissions were bovine, 39 percent porcine, 2 percent avian and 2 percent other species.

There was a large increase in the number of cases submitted as retained pending laboratory results (469 or 19 percent in FY 73). This was probably due to the recently implemented regulation concerning cooking or condemning tuberculous carcasses. It is obvious that carcasses cooked or condemned result in a considerable monetary loss for the processor or the producer or both.

Retained cases are given special treatment so that results can be reported as quickly as possible, usually by phone. This enables prompt disposition of the carcass by the meat inspector. To assure quick and accurate diagnostic results, histopathologic procedures for detecting acid-fast bacilli have been developed, evaluated and modified by our laboratory. These include fluorescent staining procedures for smears and tissue sections.

An auramine-o acridine orange combination (AOAO) was used to stain representative sections of each piece of tissue. These sections were routinely examined microscopically for acid-fast bacilli and various fungal agents. The AOAO combination has been compared with conventional fuchsin stains for detecting acid-fast bacilli and has produced equal or superior results. For this reason the fuchsin stain is used in our laboratory only in certain instances as a special confirmatory preparation.

The auramine-o stained smear (AO) techniques for the detection of acid-fast bacilli was utilized in all submissions with sufficient gross lesion material to permit this type of preparation. When acid-fast bacilli were detected by the AO smear technique, a diagnosis of "compatible" could be reported in a matter of hours, often on the same day that the submission was received. If no acid-fast organisms were found by the smear technique, additional procedures, taking an average of 1 week's time, were performed before a diagnosis could be reported.

An examination of FY 73 records indicated that 741 of 982 reports of "compatible with a diagnosis of mycobacteriosis" were detected by the AO smear technique, and comparable results (positive and negative) were obtained in 1,631 of 1,821, or 90 percent, of the cases. This is an important statistic since rapid disposition of carcasses examined only by the AO smear technique could be made with 90 percent confidence.

In addition to the fluorescent procedures described above representative sections of each tissue were stained with hematoxylin and eosin. These sections were examined microscopically for any characteristic tissue changes. Many special stains were utilized to examine for specific tissue changes or to identify various pathogenic agents.

THE ISOLATION OF MYCOBACTERIUM KANSASII FROM LYMPH NODES OF CATTLE. Jarnagin, J. L. and
Champion, M. L.

Summary

This paper describes the isolation of three strains of *M. kansasii* from bovine tissues. The isolation of this
organism from cattle provides further evidence of the pathogenicity of this organism in domestic animals.

Introduction

The isolation of atypical mycobacteria including the *M. avium* complex, from domestic animals has been
reported by numerous investigators; (6, 11, 8, 7, 1)[1] however, the isolation of members of Runyon's Group I,
particularly *M. kansasii*, has not been commonly reported from animals in the United States. *M. kansasii* has been
isolated from cattle and zoo animals (11, 8) in Europe and similar isolates have been described in swine and milk in
the United States (7, 1).

This paper describes three isolations of *M. kansasii* from tissues of cattle received for mycobacteriologic
examination.

General and Histopathologic Examination

The first isolation (NADL 716231) was made from an adult bovine animal. Suprapharyngeal, bronchial,
mesenteric, prefemoral, prescapular and postmediastinal lymph node tissues were received in 10 percent buffered
formalin for histopathologic examination and in 0.087 percent chloramine T for bacteriologic examination. The
latter compound reduces bacterial contamination.

Inflammation was seen in the prescapular and mesenteric nodes which were histopathologically suggestive of
lymphadenitis. No acid-fast bacilli were observed.

The second isolate (NADL 723150) was cultured from tissues of an 18-month-old female bovine in which the
meat inspector suspected the observed gross lesions to be due to coccidioidal granuloma.

Mediastinal lymph nodes were submitted in formalin as described above and also in saturated sodium borate
solution for bacteriologic examination. This solution, like chloramine T, acts to control gross bacterial
contamination.

Histopathologic findings revealed tissue changes suggestive of mycobacterial infection but no acid-fast bacilli
were observed.

The third isolation (NADL 727630) of *M. kansasii* was made from a 2-year-old cow that reacted to the
tuberculin test. Tissues from the bronchial and mediastinal lymph nodes described by the meat inspector as having
an unusual tissue reaction were received in formalin and sodium borate. Histopathologic evidence of lymphoid
hyperplasia was seen in the tissues but no acid-fast bacilli were observed.

Bacteriologic Examination

Each of the above tissue was processed by digestion with 5 percent papain (4) followed by concentration of
the mycobacteria with pentane (5). Slants of Lowenstein-Jensen, Stonebrink, Herrold and Middlebrook 7H10 media
were each inoculated with 0.1 ml of concentrated material and were incubated at 37° C.

Colonies of isolates 727630 and 716231 appeared on Stonebrink's medium 2 and 3 weeks following
inoculation. Isolate 723150 grew on Lowenstein-Jensen medium 5 weeks after inoculation.

Microscopically, the bacterial cells from all three isolates appeared as long acid-fast rods 2.1 - 4.0 x 0.5 when
stained by the Ziehl-Neelsen method. No cellular branching was observed. Excellent cording and crossbanding was
observed in all isolates which are characteristics commonly seen in *M. kansasii* (9).

Colonial morphology in all subcultured isolates varied from a smooth granular to a dry-flaky texture. Colonies
upon primary isolation were smooth, moist and pale yellow but darkened with age and additional exposure to light.

[1] Numbers in parentheses refer to References at the end of this report.

All isolates produced yellow-orange pigment when exposed to white light but remained white when incubated in the dark.

Carotene crystals were demonstrated in each of the isolates on 7H10 medium 3 to 4 weeks after inoculation.

Growth from all isolates appeared in approximately 14 days when subcultured at 37° C, on Lowenstein-Jensen medium. No growth was observed at room temperature or 45° C.

The biochemical characteristics of the isolates were determined by tests generally accepted for differentiating mycobacteria (3, 10, 2) and are tabulated in table 1.

Table 1.—Biochemical reactions of three M. KANSASII strains isolated from cattle

Biochemical test	Case number		
	716231	723150	727630
P/B Medium	Uniform	Uniform	Uniform
Nitrate Reduction	++++	++	++++
Catalase 20°	++++	+++	+++
Tween 80 Hydrolysis (24 hrs)	++	++	++
Arylsulfatase (3 days)	−	−	−
Tellurite Reduction	−	−	−
5% Salt Tolerance	−	−	−
INH (10 ug)	R	R	R
TCH (15 ug)......................................	R	R	R
Neotet. 1:40000	R	R	R
Streptomycin (2.0 ug)	R	S	R
Rifampin (.025 ug)	R	R	R
Niacin	−	−	−

− negative	++ moderate reaction	R = Resistant
+ weak reaction	+++ strong reaction	S = Sensitive
	++++ very strong reaction	

Discussion

The isolates described represent typical *M. kansasii* strains in that characteristic carotene crystals were observed and Tween 80 was hydrolyzed within 24 hours. These tests are definitive for this species.

M. kansasii has been isolated from humans but reported isolations from animals have been infrequent.

From the histopathologic data, it can be seen that no definite observations of acid-fast organisms were found in tissue. All tissues showed some aberrant changes - the cause of which is not known.

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COMPARISON OF WET COMPOSITING WITH STANDARD METHODS FOR DETECTING SALMONELLAE IN ANIMAL BY-PRODUCTS. *Harrington, R., Blackburn, B. O. and Murphy, C. D. (Project Report)*

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Summary

Three wet compositing techniques were compared with a standard procedure to determine the efficiency of wet compositing for the detection of salmonellae in samples of animal by-products. There was no significant difference ($\propto 0.05$) in the proportion of lots found by the four methods.

Introduction

In a previous report on dry compositing, a method was described for the examination of pooled animal by-product samples (3).[1] When the compositing methods were compared with a standard culture procedure, there was no significant difference between two of the compositing methods and standard culture. However, one compositing method appeared to be less sensitive than the standard methods.

The purpose of this study was to evaluate wet compositing as an approach for the isolation of salmonellae from samples of animal by-products.

This is a report on the comparison of a standard culture procedure (1) with wet compositing techniques for the isolation of salmonellae from animal by-products.

Materials and Methods

Animal by-product samples: Samples were obtained from rendering plants in Illinois, Indiana, Iowa, Kansas, Minnesota, Nebraska, North Dakota and South Dakota by Animal and Plant Health Inspection Service field personnel.

Mediums: Triple sugar iron (TSI) agar and lysine iron (LI) agar were prepared according to the instructions of the manufacturer.[2] Tetrathionate (TT) broth and brilliant green sulfadiazine (BGS) agar were prepared according to the methods previously described (1). Mannitol-tergitol (MT) broth was prepared by adding 20 g of tryptose,[2] 5 g of sodium chloride, 5 mg of thiamine hydrochloride, and 10 g of d-mannitol[3] per liter of distilled water. Following sterilization at 120 C for 15 minutes, 6 ml of a 10 percent solution of Tergitol Anionic 7[4] was added.

Culture Methods: A total of 600 samples of animal by-products were grouped to form 60 lots (10 samples per lot) and culture procedures were performed on one lot at a time. Each of the 10 samples in the lot was thoroughly mixed and two 30 g aliquots were removed and identified as "1" and "2." The 10 number-1 aliquots were used to form composites A_1 and A_2 while the 10 number-2 aliquots were used for the standard procedure and composite B.

The number-1 aliquot of each of the 10 samples in the lot was placed in a separate wide mouth screw cap jar and 250 ml of MT pre-enrichment broth was added. These were incubated for 18-24 hours at 37° C.

Composite A_1 was formed by removing 1 ml from each of the MT broth cultures and transferring it to a test tube containing 10 ml of double strength TT broth. Following overnight incubation at 37° C, a 5-6 mm loopful of the TT broth culture was streaked on BGS agar plates. Salmonella-type colonies appearing on the agar plates after 18-24 hours incubation were transferred to TSI and LI agar slants. Cultures producing reactions characteristic of *Salmonella sp.* were verified with polyvalent "H" antiserum according to methods previously described (2). Salmonella serotyping was performed on each isolate.

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[1] Numbers in parentheses refer to References at the end of this report.

[2] Difco Laboratories, Detroit, Mich.

[3] BBL, Division of BioQuest, Division of Becton Dickenson Co., Cockeysville, Md.

[4] Union Carbide Chemical Co., Division of Union Carbide Corporation, New York, N.Y.

Composite A_2 was similar to A_1 but different in that: (1) two-tenths ml of MT broth culture was used in place of 1 ml and; (2) single-strength TT broth was used in place of double strength TT broth.

The number-2 aliquot of each of the 10 samples in the lot was cultured according to a standard procedure (1). Composite B was prepared from TT broth cultures used in the standard procedure. A 0.1 ml amount from each of the 10 cultures was transferred to a tube containing 10 ml of single strength TT broth. After overnight incubation, the composite culture was examined as described above.

Results and Discussion

Bacteriologic examinations were performed on 600 animal byproduct samples. The results of these examinations are presented (table 1). Of the lots examined, 41 contained samples in which salmonellae were isolated from one or more by standard methods. Forty-three lots were positive by composite method A_1, 44 lots were positive by composite method A_2 and 41 lots were positive by composite method B. Three composites were negative by the three compositing techniques that contained 1 or more 30 g aliquots that were positive by standard methods.

Table 1.—Comparison of standard culture and wet compositing procedures for the detection of Salmonellae in animal by-products

Lot no.	Standard culture	Wet composites			Lot no.	Standard culture	Wet composites		
		$A_1$1	$A_2$2	B_3			$A_1$1	$A_2$2	B_3
1	5/10#	+	+	+	31	3/10	+	+	+
2	1/10	+	+	+	32	10/10	+	+	+
3	0/10	+	+	-	33	0/10	+	+	-
4	4/10	+	+	+	34	0/10	-	+	-
5	3/10	+	+	+	35	0/10	+	+	+
6	1/10	+	+	+	36	1/10	+	+	+
7	0/10	-	-	-	37	6/10	+	+	+
8	1/10	+	+	+	38	5/10	-	-	+
9	1/10	+	+	+	39	0/10	+	+	-
10	5/10	+	+	+	40	6/10	+	+	+
11	0/10	-	-	-	41	9/10	+	+	+
12	0/10	-	-	-	42	9/10	+	+	-
13	0/10	-	-	-	43	0/10	+	+	-
14	3/10	+	+	+	44	10/10	-	+	+
15	0/10	-	-	+	45	8/10	+	+	+
16	0/10	-	-	-	46	0/10	-	-	-
17	7/10	+	+	+	47	1/10	-	-	-
18	8/10	+	+	+	48	6/10	+	+	+
19	0/10	+	-	+	49	0/10	-	-	-
20	1/10	+	+	+	50	2/10	-	+	+
21	0/10	-	-	-	51	5/10	+	+	+
22	0/10	-	-	-	52	1/10	+	+	+
23	4/10	-	-	+	53	1/10	+	-	+
24	7/10	+	+	+	54	1/10	+	+	+
25	9/10	+	+	+	55	2/10	+	+	-
26	5/10	+	+	+	56	0/10	-	-	-
27	8/10	+	+	+	57	2/10	+	+	+
28	8/10	+	+	+	58	0/10	+	+	-
29	5/10	+	+	+	59	3/10	+	+	+
30	9/10	+	+	+	60	4/10	+	+	+

[1] = Pre-enrichment broth cultures subcultured in double strength tetrathionate broth.

[2] = Pre-enrichment broth cultures subcultured in single strength tetrathionate broth.

[3] = No pre-enrichment.

#= No. 30 g samples positive/No. 30 g samples examined.

+ = Salmonella isolation.

- = No Salmonella isolation.

Interestingly, salmonellae were isolated from nine composites by (one or more of) the methods A_1, A_2, or B, even though each of the 30 g aliquots comprising the composite were negative. Conversely, salmonellae were isolated from one or more individual 30-gram aliquots of eight lots even though one or more of the three composite methods were negative for these eight lots.

The results of serotyping the salmonella isolates are presented in table 2. As expected, there was not complete agreement on the serotypes found by the four techniques. However, the data are not sufficient to make statistical

Table 2.—Serotypes isolated from positive lots by standard culture and wet compositing methods

Serotype	Standard[1] culture	Wet composite		
		A_1	A_2	B
Montevideo	13	9	10	5
Infantis	5	5	5	7
Senftenberg	10	2	3	3
Oranienburg	8	4	4	0
Bredeney	6	3	2	2
Eimsbuettel	7	0	2	3
Schwarzengrund	3	2	2	3
Bareilly	1	4	2	2
Cerro	3	2	2	1
Ohio	2	2	2	2
Kentucky	3	1	1	1
Binza	4	0	1	1
Lexington	2	1	2	1
Tennessee	2	1	2	1
Thomasville	5	0	0	1
Madelia	2	1	1	1
Alachua	1	1	1	2
Albany	3	0	0	1
London	2	1	1	0
Minnesota	3	0	0	0
Cubana	2	1	0	0
Saint Paul	2	1	0	0
San Diego	2	0	1	0
Meleagridis	1	1	1	0
Siegburg	1	0	1	0
Worthington	1	0	1	0
Derby	1	0	1	0
Newington	1	0	0	1
Drypool	1	0	0	1
(3), (15), 34:g,m,s	1	0	0	1
Manhattan	1	1	0	0
Typhimurium	1	1	0	0
Newport	1	0	0	0
California	1	0	0	0
Muenchen	1	0	0	0
Give	1	0	0	0
Canoga	1	0	0	0
Illinois	1	0	0	0
Indiana	1	0	0	0
Anatum	1	0	0	0
Heidelburg	0	1	0	0
Arkansas	0	0	0	1
Carrau	0	0	0	1
Manila	0	0	0	1

[1] The standard culture method permits isolation of more than one serotype.

analysis of the differences meaningful. Each of the 10 most common serotypes previously reported (5, 6, 7) from animal by-products was found in these samples by one or more of the techniques.

A paired comparison technique for proportions (with a correction factor) as described by Snedecor and Cochran (4) was used to compare the three wet compositing methods with the standard. The results are summarized in table 3. There was no significant difference ($\alpha 0.05$) in the proportion of lots found positive by standard and compositing methods. Admittedly, the paired comparison test has low power with the small number of discrepancies involved. An examination of lot 42 and lot 44 using Fisher's Exact Probability Test (under the two assumptions that one composite is equal to 10 individual samples and that the organisms are randomly distributed throughout the original aliquot) also reveals that the composite methods will occasionally fail to detect salmonellae when they are present. Unfortunately, Fisher's Exact Probability Test has low power (or is inappropriate) in testing whether the standard culture method fails to detect salmonellae when they are present. (This is because the composite methods reveal *only* that at least one of the 10 assumed samples making up the composite is positive.) Lot 35 (where all three composite methods and 0 of 10 individual samples were positive) and lots 3, 19, 33, 39, 48 and 58 (where two of three composites and 0 of 10 individual samples were positive) indicate this as a distinct possibility. Thus while further testing may be necessary to completely resolve the issue, these findings indicate that the wet compositing procedures described herein can be used to isolate salmonellae from animal by-products with an accuracy similar to the standard methods employed.

Table 3.—*Standard and wet composite cultural results*

Standard method	Composite methods			No. of pairs		
	A_1	A_2	B	A_1	A_2	B
+	+	+	+	36	37	38
-	+	+	+	7	7	3
+	-	-	-	5	4	3
-	-	-	-	12	12	16
TOTAL				[1] 60	[2] 60	[3] 60

[1] $X^2 = 0.083$.
[2] $X^2 = 0.364$.
[3] $X^2 = 0.111$.

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Acknowledgment

THE EFFICIENCY OF DRY COMPOSITING FOR DETECTING SALMONELLAE IN ANIMAL BY-PRODUCTS.
Harrington, R., Blackburn, B. O. and Murphy, C. D. Proc. 76th Ann. Meeting USAHA, 1972, pp. 565-571. (Abstract of Published Report)

Comparisons were made between a standard method and three compositing methods for the isolation of salmonellae from rendered animal by-products. Of the 61 lots examined, 43 were positive by standard methods, 43 were positive by composite method A_1, 47 were positive by composite method A_2 and 36 were positive by composite method B.

The results of the present study indicate that composite methods A_1 and A_2 may be used to examine animal by-products for Salmonellae with an accuracy equal to the standard methods employed.

EIGHT NEW SEROTYPES OF ARIZONA ARIZONAE KAUFFMANN AND EDWARDS. Blackburn, B. O., Nelson, L. V. and Karns, V. L. International Jr. of Systematic Bacteriology, Vol. 23, No. 1, Jan., 1973, pp. 77-79. (Abstract of Published Report)

Eight new serotypes of *Arizona arizonae* Kauffmann and Edwards (syn.: *Arizona hinshawii* Ewing) were characterized biochemically and serologically. The biochemical reactions of these serotypes were typical of the genus. The antigenic formulas determined serologically were 1,4:16,17,18; 5:1,6,7; 6:1,7,8; 10a,10b:16,17,18; 19:17,20; 22:1,3,11; 24:33:28; and 28:29:31.

LACTOSE FERMENTING SALMONELLAE FROM DRIED MILK AND MILK DRYING PLANTS. Blackburn, B. O. and Ellis, E. M. Applied Microbiology, Vol. 26, No. 5, Nov., 1973, pp. 672-674. (Abstract of Published Report)

A study of 552 salmonella cultures revealed that 86 (15.6 percent) of the cultures fermented lactose. These had been isolated from dried milk products and milk drying plants. Acid and gas were produced in lactose broth. Solid media containing lactose as the key ingredient for the differential reaction were not satisfactory for recognizing salmonella colonies. No problem was encountered in selecting salmonella colonies when bismuth sulfite agar was used.

v C Virus d

SCRAPIE STUDIES ON SHEEP FROM A DEPOPULATED OKLAHOMA FLOCK. Proctor, S. J. and Jenkins, S. J. (Project Report)

Introduction

Scrapie was diagnosed in a small flock (24) of Oklahoma sheep. Many of the remaining sheep in the herd were losing wool. Flock depopulation was initiated and brain tissue was obtained to determine if other sheep in the herd were infected with scrapie. Three different fixatives were compared on these brains. Previous studies suggested that formalin or formal-glutaraldehyde were preferred over Zenker's or glutaraldehyde fixatives.

Materials and Methods

After euthanasia, the brain was removed from each sheep and the frontal lobes were removed and frozen. The remainder of the brain was divided along the median plane, and each half was randomly placed in one of three fixatives:
A. Neutral-buffered 10 percent formolsaline (phosphate buffered).
B. 10 percent formalin in 0.067 M phosphate buffer plus 7.5 percent sucrose.
C. 1.25 percent glutaraldehyde and 5 percent formalin at 440 mOsM of phosphate buffer and saline.
Paraffin and frozen sections were prepared from the medulla, pons, mesencephalon, and thalamus and stained with azure-eosin-hematozylin and Cajal's gold-sublimate technique, respectively.

Results

Typical scrapie lesions could not be found in any brain sections examined. An occasional single vacuolated neuron was found in a few sections; but since these vacuoles were located in the periphery of the cytoplasm and did not push the nucleus eccentrically, they were not considered indicative of scrapie.

When stained with azure-eosin-hematoxylin, there was no significant increased preservation of tissue with fixatives B or C when compared to A (our routine fixative). Fixative C (glutaraldehyde-formalin) stained poorly with Cajal's gold-sublimate. There was no significant difference between A and B—the formalin fixatives.

Discussion

No lesions indicative of scrapie could be found in any of the sheep. Most of the vacuoles found were peripheral and could have been due to handling of the brain after death. More centrally located vacuoles were found predominantly in the older sheep (7-10 years old). No more than four vacuoles in one section or total brain were observed.

Although there was an increased number of astrocytes seen in many of the brains, the astrocytic processes were thin, short, and uniform. They did not resemble the large irregular astrocytic processes caused by scrapie. There was no indication of an increased number of reactive astrocytes with increased age.

Preservation and stainability of microscopic sections were used as criteria to evaluate each fixative. Tissues held in fixative A (our routine fixative) were preserved and stained as well as or better than tissues in the other two fixatives. It was decided to continue to use "A" as the fixative of choice.

SCREWWORM

ATTRACTANCY OF INOCULATED AND INCUBATED BOVINE BLOOD FRACTIONS TO SCREWWORM FLIES (DIPTERA: CALLIPHORIDAE): ROLE OF BACTERIA DeVaney, J. A., Eddy, G. W., Ellis, E. M. and Harrington, R., Jr. J. Med. Ent. Vol. 10, No. 6, Dec., 1973. (Abstract of Published Report)

Olfactometer tests in the laboratory with bacteria-contaminated citrated whole bovine blood, bacteria-inoculated sterile citrated whole blood, defibrinated blood, and blood plasma indicated that the attractancy of these materials to screwworm flies, *Cochiomyia hominivorax* (Coquerel), resulted from the bacteria and/or from the compounds produced by them. Aseptically drawn blood incubated at 37° C. for 7 days was no more attractive than unincubated blood held at 5° C. Ranking of attractiveness was bacteria-inoculated sterile defibrinated blood > bacteria-inoculated citrated whole blood > bacteria-inoculated plasma > sterile-incubated defibrinated blood-distilled water.

VESICULAR STOMATITIS

b[Viral]"

SURVEY FOR VESICULAR STOMATITIS INFECTION IN GEORGIA WILD MAMMALS, Jenney, E., Hayes, F. and Brown, C. (Project Report)

Summary

Four hundred and nineteen serums from small feral mammals collected in Georgia during 1964 and 1965 were tested for New Jersey type VS virus neutralizing antibodies. Antibodies were found in 4 of 105 raccoons, 2 of 115 opossums, and 1 of 9 gray squirrels. Serums from 15 red foxes, 90 gray foxes, 23 skunks, 10 bobcats, 3 coyotes and 49 miscellaneous animals were negative for antibodies.

Introduction

There have been several reports describing the distribution of vesicular stomatitis (VS) antibodies in mammals of the southeastern United States (4, 5)[1] and Panama (12, 13). This report contains additional information regarding New Jersey type VS antibodies detected in wild mammals during a Georgia survey. Indiana type VS is not included in the survey since it has not occurred in domestic animals in the southeastern United States (3, 6, 7, 9).

During 1963 there was an extensive outbreak of New Jersey VS involving livestock of 23 counties in west central Georgia (6). Cases were also diagnosed in five counties scattered throughout the remainder of the State. The incidence declined in 1964 with VS confirmed in only five counties. Clinical VS has not been reported in Georgia since 1964. In 1970, however, subclinical VS was confirmed in domestic and wild mammals found on one of its coastal islands (10).

Materials and Methods

Serums were collected during 1964 and 1965 by the Southeastern Cooperative Wildlife Disease Study and made available for VS serology. Animals were caught in live traps, anesthetized and blood samples were collected from the heart using 10 ml. syringes with 20 guage needles. Neutralization tests were performed on 419 wildlife serums in 8-day embryonated chicken eggs using a method previously described in Geleta and Holbrook (2). Tests were run for New Jersey type VS.

Results

Serums from 4 of 105 raccoons *(Procyon lotor)*, 2 of 115 opossums *(Didelphis marsupialis)* and 1 of 9 gray squirrels *(Sciurus carolinensis)* had New Jersey type VS antibodies. The serums of other animals were negative; these include the predatory species, 15 red foxes *(Vulpes fulva)*, 90 gray foxes *(Urocyon cinereoargenteus)*, 23 skunks *(Mephitis mephitis)*, 10 bobcats *(Lynx rufus)*, 6 housecats *(Felis catus)* and 3 coyotes *(Canis latrans)*. Serums of 29 cotton rats *(Sigmodon hispidus)* and 9 rabbits *(Sylvilagus floridanus)* were also negative. An accompanying map shows the distribution within the State.

The animals tested in each county are shown in table 1.

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[1] Numbers in parentheses refer to References at the end of this report.

Table 1.—Summary of animals tested

(Numerator—Number of positive animals; Denominator—Total number of animals tested)

Species		Brantley	Brooks	Burke	Chatham	Clarke	Crisp	Dough-erty	Glynn	Houston	Loundres	McIntosh	Pierce	Screven	Telfair	Thomas	Turner
												Counties					
Raccoons		0/5	1/15	0/6			0/12		0/4		0/18	3/8	0/9	0/7			0/21
Gray fox.		0/10	0/20	0/8			0/9		0/1		0/10	0/4	0/7	1/3	0/3		0/21
Opossums . . .		0/10	0/12	0/2			0/17		0/7		0/15	0/7	0/11				1/23
Red fox		0/2	0/1			0/1	0/1			0/5	0/1		0/6		0/1		0/3
Striped skunk.		0/1	0/9				0/5				0/2		0/4				0/1
House cats . . .			0/2	0/1			0/1				0/1				0/1	0/3	0/2
Gray squirrel .			0/1								0/1	1/1					0/1
Black rat					0/1			0/1		0/5							
Cotton rat . . .					0/1		0/1		0/2		0/2				0/6	0/16	0/3
Bobcat						0/1	0/2					0/1					0/1
Coyotes							0/1										0/1
Fox squirrel . .										0/2							
House mice . .											0/4				0/1		0/2
Cottontail rabbits							0/2										

48

Discussion

The sampling in this current Georgia group occurred 1 year after a peak of infection was observed. There was a better chance of finding antibodies in animals with longer life cycles than in animals with short life cycles.

New Jersey VS outbreaks occurred in Texas cattle and horses during periods of high cotton rat density in 1949 and 1959 (1, 11). There are reports of sudden decreases of cotton rat density and the finding of dead carcasses (1, 8). From our work with cotton rats (unpublished) it appears likely that death would occur following infection with NJ type VS so few survivors would be found with antibodies to the virus. It is not known if cotton rats become infected with New Jersey type VS in nature. This information would be helpful in establishing or eliminating New Jersey VS as a cause of cotton rat die-offs. Observations concerning rodent die-offs or their population density before and after VS outbreaks would be helpful in determining their role, if any, in VS epizootiology.

All of the animals in which New Jersey type antibodies were found in this current study were semi-arboreal and eat plants as part of their diet. In addition the opossums and raccoons eat carrion and small wildlife. It appears that the raccoons and opossums could be infected by eating carrion or infected rodents, reptiles or other forms of wildlife. Squirrels, one of which was positive, have arboreal habits and their diet consists of nuts, plants and a few insects. An arthropod source of infection must be considered for this positive squirrel.

Hanson and Karstad (5) found New Jersey type VS antibodies in 35 (45 percent) of 77 raccoons collected about 1955 in southern Georgia. Raccoons inoculated experimentally by Hanson and Brandley (4) developed subclinical infections. New Jersey type VS antibodies were also found in the serums of 3 (37 percent) of 8 bobcats, 46 (79 percent) of 58 feral swine, and 12 (60 percent) of 20 deer. New Jersey VS has been diagnosed in Georgia livestock during the years 1952 - 1959. Our findings of VS antibodies in opossums in general parallels findings in Panama. Tesh (13) in Panama found NJ neutralizing antibodies in 8 of 160 common opossums *(Didelphis marsupialis)* and antibodies in 16/272 other genera of opossums not found in the United States; 20 raccoons were negative for NJ type VS antibody. Srihongse (12) reported finding Indiana type antibodies in 7 of 40 common opossums but did not list New Jersey tests. Indiana type VS is endemic and predominant in Panamanian tropical jungle areas which do not contain livestock. The ecology of the two virus types appears to be different.

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DISTRIBUTION OF 419 GEORGIA MAMMAL SERUMS EXAMINED FOR NEW JERSEY VESICULAR STOMATITIS ANTIBODIES (1964-1965 COLLECTION)

NUMERATOR - NUMBER OF POSITIVE ANIMALS
DENOMINATOR - TOTAL NUMBER OF ANIMALS TESTED
R - RACCOON
S - SQUIRREL
P - OPOSSUM

BIOLOGICAL SAFETY

BIOLOGICAL SAFETY IN THE VETERINARY DIAGNOSTIC VIROLOGY LABORATORY. Pearson, J. E.

Biological safety and security is extremely important in the veterinary diagnostic virology laboratory. The specimens received may contain agents known to be infectious to animals but the potential effect on humans is unknown. This laboratory is often the first laboratory involved in an exotic disease outbreak.

There are three areas to be considered when discussing biological safety.

1. Protection of the laboratory worker. The danger of human infection should always be considered when any potentially hazardous specimen is received, e.g., an encephalitis case. The hazard to all laboratory personnel including mail handlers, janitors, maintenance, etc., must be considered.

2. Containment of viral agents. Specimens received may contain infectious agents that are exotic to that country and should not be released into the environment. This has taken on new importance recently with the hog cholera eradication program, Venezuelan equine encephalitis in Mexico and velogenic viscerotropic Newcastle in California.

3. Protection of diagnostic procedures from contamination. A diagnostic virology laboratory as compared to a research laboratory will usually have a variety of agents present in a relatively small area. Therefore it is important that extra precautions be taken to prevent cross contamination.

A virus laboratory has several problems associated with its specialized function. The use of antibiotics in cultures and preparation of specimens helps to control possible bacteria infections and contamination. However, many viral isolation procedures require grinding tissues which can produce viral laden aerosols. It is possible a human infection may be produced before the causative virus is identified.

Each laboratory has unique problems so a set of rules that are applicable to all cannot be developed. The laboratory supervisor has the ultimate responsibility for biological safety. He must often consider the safest technique as compared to the technique that would be the most efficient. Even though a variety of diagnostic specimens may be received there is usually adequate history to make a decision as to what safety precautions are required. The supervisor should be free to impose more stringent or lenient restrictions as the situation demands. Often in a diagnostic laboratory only a relatively small area is needed for viral isolation and identification. The biological security in this area should be very stringent as compared to the area where serology with inactivated reagents is being conducted.

There are several good references available that describe methods for handling hazardous viral material, particularly human pathogens, and on developing laboratory designs (1, 2, 3, 4, 5, 7).[1]

Following are some general precautions and security methods which have been applied within Diagnostic Virology, Veterinary Services Diagnostic Laboratory, Ames, Iowa. As Chlamydia and Rickettsia agents are propagated on the same host systems as viruses they are included.

Air Handling Systems

Control of aerosols, particularly as produced by tissue grinding techniques such as blendors, is important. The most desirable method is to have the laboratory under negative pressure as compared to the corridors. Air is not recirculated but exhausted to the outside atmosphere through biological filters. Isolating the virology laboratory in a separate building will add a dilution effect to the air when it is released. It should still be passed through biological filters because air currents can carry a relatively high concentration of virus in one direction.

An alternate method is the use of biosafety cabinets or vertical laminar flow hoods in a room with recirculating air. The biosafety cabinet is the safest type. It draws air into the hood from the room at 100 linear feet per minute and exhausts through biological filters. This poses the additional hazard of drawing viral agents into the

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[1] Numbers in parentheses refer to References at the end of this report.

hood from the room. The vertical laminar flow hood[2] draws only 10-20 percent of the air used from the room. The remainder of the air is recirculated through biological filters. A vertical laminar flow hood provides about the same protection as a closed cabinet that does not have glove ports. Any air control system can give a false sense of security if it is not selected carefully and checked continuously. The selection and use of air control systems are covered in more detail in references (2, 4, 5). When working with Venezuelan equine encephalitis, Chlamydia agents, and other hazardous agents we do all the work inside a vertical laminar flow hood in a laboratory under negative pressure.

Movement of Personnel

Extraneous persons should be discouraged from entering any laboratories where virus isolation procedures are being carried out. If a hazardous or exotic virus is suspected, the supervisor should allow only essential personnel into the laboratory. It may also be desirable to require all personnel to take a decontaminating shower before leaving the laboratory. If a hazardous viral agent is being used on a routine basis as part of a test procedure, it may be advisable to keep the laboratory closed at all times. Whenever possible, an avirulent or killed agent should be used in place of a hazardous agent. The laboratory surfaces can be disinfected and the laboratory opened to other personnel when low-risk agents are being used. Signs should be posted outside the laboratory describing the hazard and the procedure for admittance.

Movement of Equipment and Supplies

All specimens, equipment and other material that may have been in contact with viral agents should be disinfected or incinerated. Autoclaving is the ideal method of disinfection. For certain equipment and supplies, other methods of sterilization may be used such as formaldehyde vapor (6), ethylene oxide or ultraviolet sterilization. The sewage leaving the laboratory should be treated to kill pathogenic agents. It is not advisable to flush infected material down drains even if sewage is treated. Due to aerosol production, garbage disposals are not recommended.

Laboratory Clothing

Personnel working in a diagnostic virology laboratory should be provided with at least laboratory coats and preferably a complete set of clothing. When working with hazardous agents, laboratory clothing should be left within the laboratory and autoclaved out. As a minimum precaution, laboratory coats should not be worn outside the laboratory and other laboratory clothing covered with a different type of laboratory coat. Using a different color of clothing within the laboratory helps prevent inadvertent use of laboratory clothing outside the laboratory.

General Laboratory Procedures

The following is a list of procedures which we use to help maintain biological security. Most of these are dependent upon the laboratory worker so each individual should understand the basic principles and importance of biological security.
1. Wash hands frequently—especially between procedures or before eating, drinking and smoking.
2. Disinfect table surface after working with each specimen.
3. A minimum of reference material and supplies should be kept in viral isolation areas.
4. Wash and disinfect floors as needed.
5. Immediately clean all viral material that is spilled.
6. Use tightly sealed containers for centrifuging.
7. Do not eat or smoke in the same area and at the same time as viral material is being processed.
8. Stock reagents, cleaning supplies, brooms, etc., should not be removed from the laboratory without disinfection.
9. Laboratories should be insect- and rodent-free.
10. Pipettes should be plugged with cotton and there should be no mouth-pipetting. It is advisable to use bulbs for all pipetting in a diagnostic virology laboratory.

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[2] The Baker Company, Inc., 106 Granite Street, Biddeford, Maine 04005.

Immunization

Vaccines are available for several agents to which diagnostic virology personnel might be exposed. The Lab Safety Manual for the Center for Disease Control has published detailed recommendations on the use of these vaccines which should be followed by any laboratory doing diagnostic virology. It is recommended that those individuals working with rabies virus be vaccinated and then checked to insure they have a neutralizing antibody response.

Preparation of Susceptible Cell Culture

In diagnosing viral disease it is mandatory that cell cultures are not contaminated with non-cytopathogenic viruses. If possible cell cultures should be prepared in a separate laboratory by different personnel. If only one laboratory is available, cell cultures should be prepared in a separate room with a different air supply or in a clean laminar flow hood. As a last choice a clean bench area may be used in the laboratory at a time when virus infected material is not being processed. If primary cultures are used, the tissues should be from healthy animals and cultures should be checked for latent virus infection before use. If serum is used in the culture, it should be filtered and checked for virus before use. Fetal bovine serum is often contaminated with bovine virus diarrhea virus.

Viral Isolation in Experimental Animals

In vitro isolation, techniques should be used whenever possible. Some procedures, such as arbovirus isolation, require animal inoculation. All the precautions that have been described for laboratory work must also be used for animal areas. Limiting personnel movement and immunizations are of particular importance. Even more stringent control must be used, because the animals may excrete large amounts of virus. Exhaust-air from animals cages or rooms should be passed through biological filters. All droppings, bedding, etc. should be incinerated. If post mortem of the animal is required, precautions should be taken to prevent aerosols. All dead animals should be incinerated. If they must be moved to another building for incineration, closed disinfected containers should be used. It may be possible to autoclave the tissues from small animals and then take them elsewhere for disposal. All potentially infected tissues or animals must be disposed of within the laboratory. Rendering companies should not receive tissues from infected laboratory animals.

In summary, the supervisor of a diagnostic virology laboratory must continually maintain the biological safety and security required for the procedures being accomplished. He must insure that workers in the laboratory are protected against infection with human pathogens. Potentially hazardous or exotic viral agents must not be released into the environment. To obtain dependable results, virus isolation procedures must be protected from laboratory contamination.

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MISCELLANEOUS

Feed was withheld from four ponies for 9 days, resulting in an average body weight loss of 26.1 kg. Blood samples were collected each morning during the fast, serum was prepared, and serum constituents were determined. Values during fasting were compared with prefasting and refeeding values. During fasting, body temperature, plasma glucose, calcium, chloride, total protein, albumin, α_2-globulin, β-globulin, γ-globulin, α-lipoprotein, glutamic oxalacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), ornithine carbamyl transferase (OCT), alkaline phosphatase (ALP), and creatine phosphokinase (CPK) concentrations were maintained at or near prefasting values. Cholesterol, phospholipid, free fatty acids (FFA), esterified fatty acids (EFA), triglycerides, pyruvate, lactate, β-lipoprotein, and α_1-globulin significantly increased in plasma, whereas urea nitrogen, magnesium, and phosphorus decreased during the fasting period. All plasma constituent values returned to prefasting values by the sixth day of refeeding.

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